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The Macfarlane Burnet Centre for Medical Research Limited;
Commonwealth Scientific and Industrial Research Organisation and
The Australian National University

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Recombinant Viral Constructs and Methods Relating Thereto"

The invention is described in the following statement:

RECOMBINANT VIRAL CONSTRUCTS AND METHODS RELATING THERETO

FIELD OF THE INVENTION

The present invention relates generally to recombinant viral constructs expressing a protective antigen together with a cytokine and to vaccine compositions comprising same. In particular, the present invention is directed to a recombinant viral construct capable of inducing a protective immune response to an HIV antigen and, most particularly an HIV-1 antigen. The present invention is useful, *inter alia*, in the therapeutic and prophylactic treatment of HIV.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

There is currently no effective method of treating HIV infection. Current treatment strategies can suppress plasma HIV-1 RNA levels to very low levels, however latently infected cells harbouring HIV-1 DNA remain detectable and viral resistance and relapse is common [1,2]. Treatment-induced reductions in HIV-1 levels results in a loss of antigenic stimulus for effective immune responses. HIV-specific cytotoxic T lymphocyte (CTL) responses, thought to be a critical effector mechanism in the control of HIV-1, decline to low levels following effective anti-HIV therapy [3].

Previous trials of therapeutic HIV-1 vaccines have shown that it is possible to stimulate anti-HIV immune responses in HIV-1 infected individuals, but no clinical benefit has been demonstrated [4-6]. Prior studies have used protein-based HIV-1 vaccines incapable of inducing CTL responses or vaccinated individuals with substantial levels of replicating HIV-1. Even moderate levels of replicating HIV-1 results in a loss of HIV-specific $CD4^+$ T-helper (Th) responses which are required to initiate and sustain an effective CTL

response [7].

Additionally, no preventative HIV vaccines currently exist. Although simple recombinant avipox vaccines (without co-expression of cytokines) can induce CTL responses in a proportion of human and non-human primate subjects, the response is often weak, transient, or non-existant. There is a need for more reliable vaccine vectors for the induction of HIV specific CTL and Th responses.

In work leading up to the present invention, the inventors have determined that the magnitude and phenotype of the specific immune response to HIV can be enhanced by vaccination with a recombinant fowl pox virus construct comprising both an HIV gag/pol encoding nucleic acid molecule and a cytokine encoding nucleic acid molecule, in particular, IFN-γ.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

One aspect of the present invention provides a recombinant viral construct comprising an avipox viral vector or functional derivative thereof which incorporates a first nucleic acid molecule encoding one or more HIV antigens or derivatives thereof and a second nucleic acid molecule encoding a cytokine or functional derivative thereof wherein said recombinant viral construct is effective in inducing, enhancing or otherwise stimulating an immune response to said HIV antigen.

Another aspect of the present invention there is provided a recombinant viral construct comprising an avipox viral vector or functional derivative thereof which incorporates a

first nucleic acid molecule encoding one or more HIV-1 antigens or derivatives thereof and a second nucleic acid molecule encoding a cytokine or functional derivative thereof wherein said recombinant viral construct is effective in inducing, enhancing or otherwise stimulating an immune response to said HIV-1 antigen.

Yet another aspect of the present invention provides a recombinant viral construct comprising an avipox viral vector or functional derivative thereof which incorporates a first nucleic acid molecule encoding HIV-1 Gag and Pol or derivatives thereof and the second nucleic acid molecule encoding a cytokine or functional derivative thereof wherein said recombinant viral construct is effective in inducing, enhancing or otherwise stimulating an immune response to said Gag and Pol.

Still yet another aspect of the present invention provides a recombinant viral construct comprising an avipox viral vector or functional derivative thereof which incorporates a first nucleic acid molecule encoding HIV-1 Gag and Pol or derivatives thereof and a second nucleic acid molecule encoding interferon-γ or functional derivative thereof wherein said recombinant viral construct is effective in inducing, enhancing or otherwise stimulating an immune response to said Gag and Pol.

A further aspect of the present invention provides a recombinant viral construct, comprising a fowl pox viral vector or functional derivative thereof which incorporates a first nucleic acid molecule encoding HIV-1 Gag and Pol or derivatives thereof and a second nucleic acid molecule encoding interferon-γ of functional equivalent thereof wherein said recombinant viral construct is effective in inducing, enhancing or otherwise stimulating an immune response to said Gag and Pol.

Another further aspect of the present invention relates to a vaccine comprising a recombinant viral construct which construct comprises a avipox viral vector or functional derivative thereof which incorporates a first nucleic acid molecule encoding one or more HIV-antigens or derivatives thereof and a second nucleic acid molecule encoding a

cytokine or functional derivative thereof wherein said recombinant viral construct is effective in inducing, enhancing or otherwise stimulating an immune response to said HIV-antigens.

Still another further aspect of the present invention provides a vaccine comprising a recombinant viral construct which construct comprises an avipox viral vector or functional derivative thereof which incorporates a first nucleic acid molecule encoding HIV-1 Gag and Pol or derivatives thereof and a second nucleic acid molecule encoding interferon γ or functional derivative thereof wherein said vaccine is effective in inducing, enhancing or otherwise stimulating an immune response to said Gag and Pol.

Still yet another further aspect of the present invention provides a method of inducing, enhancing or otherwise stimulating an immune response, in a mammal, to one or more HIV-antigens said method comprising administering to said mammal an effective amount of a vaccine as hereinbefore defined.

In another aspect there is provided a method for the treatment or prophylaxis of HIV infection or AIDS in a mammal said method comprising administering to said mammal an effective amount of a vaccine as hereinbefore defined.

Yet another aspect of the present invention provides an agent useful for inducing, enhancing or otherwise stimulating an immune response to an HIV-antigen said agent comprising a recombinant viral construct as hereinbefore defined.

Still yet another aspect of the present invention provides a pharmaceutical composition for use in inducing, enhancing or otherwise stimulating an immune response to an HIV antigen in a mammal comprising a recombinant viral construct as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents. The composition may also comprise the recombinant viral construct and a known antiviral compound or molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the construction of FPVgag/pol-IFNα.

Figure 2 is a schematic representation of the construction of FPVgag/pol.

Figure 3 is a graphical representation of the safety of FPVgag/pol-IFNγ immunisation. **A**. No significant fever was documented following FPVgag/pol-IFNγ vaccination of macaques. Animals M9 and M10 (◆,■) received FPVgag/pol-IFNγ 10^8 PFU IM, animal M7 (△) received FPVgag/pol, and animals M2, M3, M4 and M5 (○) were unvaccinated controls. **B**. No change in T cell or monocyte counts was observed following FPVgag/pol-IFNγ vaccination of macaques. PBMC obtained from animals vaccinated with FPVgag/pol-IFNγ (M9, M10) or FPVgag/pol (M7) were stained for $CD4^+$ T cells, $CD8^+$ T cells and $CD14^+$ monocytes prior to vaccination (△, 6 times over 8 months prior to vaccination, mean±SD shown) on the day of vaccination (○) and following vaccination (■, weekly for 4 weeks following vaccination, mean±SD shown).

Figure 4 is a graphical representation of enhanced gag-specific Th1 response following FPVgag/pol-IFNγ vaccination of macaques. **A**. T cell proliferative response to p24 antigen was measured serially following 2 vaccinations (arrows) of macaques with FPVgag/pol-IFNγ (animals M9, M10, solid and hatched bars) or FPVgag/pol vaccinations of macaques (animal M7, open bars). **B**. Secretion of IFN-γ and IL-4 by PBMC in response to recombinant HIV-1_{SF2}p24 protein stimulation obtained before and after the first FPV vaccination. FPVgag/pol-IFNγ vaccinated macaques (M9 and M10) and a FPVgag/pol immunised animal (M7) is shown.

Figure 5 is a graphical representation of enhanced Gag/pol specific CTL response following FPVgag/pol-IFNγ vaccination. Quantification of CTL precursors to Env and Gag/pol antigens was analysed following FPV vaccinations. CTL frequencies were assessed following initial and booster FPV vaccinations (arrows). Recognition of control

targets expressing vaccinia antigens alone have been subtracted.

Figure 6 is a photographic representation of Gag/pol specific antibodies are enhanced following FPVgag/pol vaccination. Western blotting of serial plasma (1:100 dilution) from animals M7, M9 and M10 following FPV vaccinations (arrows). Strips are labelled with weeks prior to or following the first and second vaccinations. Negative and positive controls represent uninfected and HIV-1 infected humans respectively.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated, in part, on the determination that the immune response to HIV, and in particular HIV-1, can be enhanced when vaccination is performed utilising a recombinant viral construct comprising both a nucleic acid molecule encoding one or more protective HIV antigens, such as gag/pol, and a nucleic acid molecule encoding a cytokine, such as IFN-γ.

Accordingly, one aspect of the present invention provides a recombinant viral construct comprising an avipox viral vector or functional derivative thereof which incorporates a first nucleic acid molecule encoding one or more HIV antigens or derivatives thereof and a second nucleic acid molecule encoding a cytokine or functional derivative thereof wherein said recombinant viral construct is effective in inducing, enhancing or otherwise stimulating an immune response to said HIV antigen.

Reference herein to "HIV" should be understood as including reference to any HIV strain including homologues and mutants. In a particularly preferred embodiment said HIV is HIV-1.

According to this preferred aspect of the present invention there is provided a recombinant viral construct comprising an avipox viral vector or functional derivative thereof which incorporates a first nucleic acid molecule encoding one or more HIV-1 antigens or derivatives thereof and a second nucleic acid molecule encoding a cytokine or functional derivative thereof wherein said recombinant viral construct is effective in inducing, enhancing or otherwise stimulating an immune response to said HIV-1 antigen.

Reference to "inducing, enhancing or otherwise stimulating" an immune response to an HIV-1 antigen should be understood as stimulating or facilitating the stimulation of a specific immune response. The specific immune response may be a T-cell and/or humoral response which is directed to any one or more peptides or epitopes, respectively, of the

HIV antigen encoded by the nucleotide sequence comprising the recombinant viral construct of the present invention. Preferably, the immune response is a Th-1 and CTL response. However, even where an immune response is skewed to a Th-1 type response, some degree of antibody generation may nevertheless occur.

Reference to "HIV antigen or derivative thereof" should be understood as a reference to any component of HIV or derivative thereof. Said component may be a peptide, polypeptide, protein or non-proteinaceous fragment such as a carbohydrate. It should be understood that the antigen may comprise one or more sites in respect of which a specific immune response is stimulated. For example, processing of the antigen by an antigen presenting cell may result in the production of one or more peptides which are co-expressed with MHC class II and which stimulate specific T helper cells. Similarly, the processing and co-expression of said peptides together with MHC class I may lead to the stimulation of one or more specificities of T cytotoxic cells. Said antigen may also comprise one or more epitopes to which a humoral immune response may be directed. Said epitope may be a linear or a conformational epitope. Where the epitope is a linear epitope, folding of the expressed antigen into its native conformation may not be required to achieve the stimulation of a specific humoral response directed to that epitope. Said antigen may for example comprise only one epitope and may take the form of a hapten. However its co-expression with a cytokine, in accordance with the present invention, may be sufficient to render said hapten immunogenic and therefore suitable for use in the present invention. Accordingly, it should be understood that reference to stimulating a response to an HIV "antigen" should be understood as a reference to the stimulation of specific immune cells (i.e. T cells and/or B cells) which are directed to one or more sites of the HIV antigen. Examples of antigens suitable for use in the present invention include, but are not limited to, one or more of the molecules encoded by the HIV viral genes *gag*, *pro*, *pol* and *env*. The expression product of each gene is given the same name, but in normal type with the first letter capitalized. Preferably said HIV antigens are the HIV-1 Gag and Pol molecules or derivatives thereof.

According to this preferred embodiment there is provided a recombinant viral construct comprising an avipox viral vector or functional derivative thereof which incorporates a first nucleic acid molecule encoding HIV-1 Gag and Pol or derivatives thereof and the second nucleic acid molecule encoding a cytokine or functional derivative thereof wherein said recombinant viral construct is effective in inducing, enhancing or otherwise stimulating an immune response to said Gag and Pol.

Reference to "cytokine" is a reference to any cytokine or derivative thereof which is capable of modulating the stimulation of an immune response. For example, said cytokine may induce, up-regulate, enhance or maintain an immune response. Particularly preferred cytokines are those which either support a Th1 response, a CTL response or skew a response towards a Th1 type response. For example, IL-2 and γ-interferon or functional equivalents thereof. Preferable said cytokine is γ-interferon.

Accordingly, in a particularly preferred embodiment there is provided a recombinant viral construct comprising an avipox viral vector or functional derivative thereof which incorporates a first nucleic acid molecule encoding HIV-1 Gag and Pol or derivatives thereof and a second nucleic acid molecule encoding interferon-γ or functional derivative thereof wherein said recombinant viral construct is effective in inducing, enhancing or otherwise stimulating an immune response to said Gag and Pol.

Avipox viral vectors suitable for use in the present invention may comprise the whole or part of any avipox virus or derivative thereof. The present invention should be understood to include derivatives such as modified virus, for example virus which has been attenuated. Examples of avipox viruses suitable for use in the present invention include, but are not limited to, fowl pox virus and canary pox virus. Preferably said avipox virus is fowl pox virus.

In a most preferred embodiment there is provided a recombinant viral construct, comprising a fowl pox viral vector or functional derivative thereof which incorporates a

first nucleic acid molecule encoding HIV-1 Gag and Pol or derivatives thereof and a second nucleic acid molecule encoding interferon-γ of functional derivative thereof wherein said recombinant viral construct is effective in inducing, enhancing or otherwise stimulating an immune response to said Gag and Pol.

Derivatives include fragments, parts, portions, chemical equivalents, mutants, homologs, mimetics from natural, synthetic or recombinant sources including fusion proteins. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences including fusions with other peptides, polypeptides or proteins.

The derivatives include fragments having particular epitopes or parts of the entire protein fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules. For example, the vector or derivative thereof may be fused to a molecule to facilitate its entry into a cell. Derivatives of nucleic acid sequences may be derived from single or multiple nucleotide substitutions, deletions and/or additions including fusion with other nucleic acid molecules. The derivatives of the nucleic acid molecules of the present invention include oligonucleotides, PCR primers, antisense molecules and fusion of nucleic acid molecules.

The nucleic acid molecule suitable for use in the present invention may be DNA or RNA. Preferably said nucleic acid molecule is DNA. Reference to the cytokine or HIV antigen encoded by a nucleic acid molecule is a reference to the expression product of said nucleic

acid molecule.

Without limiting the present invention to any one theory or mode of action, it is thought that administration of the recombinant construct of the present invention enhances the phenotype and magnitude of the HIV specific T-cell response. It may also result in expansion of the T-cell repertoire directed to the T-cell antigen comprising the construct of the present invention. A protective immune response against HIV-1 (specifically against the HIV-1 antigen comprising the construct) is therefore stimulated in individuals administered the recombinant viral construct of the present invention.

Accordingly, another aspect of the present invention relates to a vaccine comprising a recombinant viral construct which construct comprises a avipox viral vector or functional derivative thereof which incorporates a first nucleic acid molecule encoding one or more HIV-antigens or derivatives thereof and a second nucleic acid molecule encoding a cytokine or functional derivative thereof wherein said recombinant viral construct is effective in inducing, enhancing or otherwise stimulating an immune response to said HIV-antigens.

Preferably said HIV-antigens are HIV-1 Gag and Pol. Even more preferably said cytokine is interferon-γ.

According to this preferred embodiment there is provided a vaccine comprising a recombinant viral construct which construct comprises an avipox viral vector or functional derivative thereof which incorporates a first nucleic acid molecule encoding HIV-1 Gag and Pol or derivatives thereof and a second nucleic acid molecule encoding interferon γ or functional derivative thereof wherein said vaccine is effective in inducing, enhancing or otherwise stimulating an immune response to said Gag and Pol.

Most preferably said avipox viral vector is a fowl pox viral vector.

Still another aspect of the present invention provides a method of inducing, enhancing or otherwise stimulating an immune response, in a mammal, to one or more HIV-antigens said method comprising administering to said mammal an effective amount of a vaccine as hereinbefore defined.

Reference to "mammal" should be understood to all mammals including primates (e.g. humans, monkeys), livestock animals (e.g. sheep, cows, horses, donkeys, goats, pigs), laboratory test animals (e.g. rats, guinea pigs, rabbits, hamsters), companion animals (e.g. dogs, cats), and captive wild animals (e.g. kangaroos, deer, foxes). Preferably, said animal is a primate.

The method of the present invention is useful in the treatment and prophylaxis of HIV infection and AIDS. For example, the vaccine of the present invention may be administered into subjects known to be infected with HIV in order induce an immune response against HIV thereby preventing the onset of AIDS. Alternatively, the method of the present invention may be used to reduce serum viral load, to alleviate AIDS symptoms or to induce immunity in mammals thought to be at risk of HIV infection.

The method of the present invention may be particularly useful either early in HIV infection to prevent the establishment of a viral reservoir or for a period after exposure to a possible source of HIV infection.

Accordingly, in another aspect there is provided a method for the treatment or prophylaxis of HIV infection or AIDS in a mammal said method comprising administering to said mammal an effective amount of a vaccine as hereinbefore defined.

In accordance with this method, the vaccine of the present invention may be co-administered with a known anti-viral compound or molecule. Such compounds or molecules include, but are not limited to, reverse transcriptase inhibitors (for example, Zidovudine or 3TC) or protease inhibitors (for example, Indinavir). By "co-administered"

is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the vaccine and the known anti-viral compound or molecule. The vaccine and the known anti-viral compound or molecule may be administered in any order.

Routes of administration include but are not limited to intravenously, intraperitoneally, subcutaneously, intracranially, intradermally, intramuscularly, intraocularly, intrathecally, intracerebrally, intranasally, infusion via i.v., drip, and implant. Intramuscular routes are particularly preferred.

The present invention further extends to the use of the subject recombinant viral construct in the manufacture of a medicament for the therapeutic or prophylactic treatment of HIV infection.

Yet another aspect of the present invention provides an agent useful for inducing, enhancing or otherwise stimulating an immune response to an HIV-antigen said agent comprising a recombinant viral construct as hereinbefore defined.

Yet another aspect of the present invention provides a pharmaceutical composition for use in inducing, enhancing or otherwise stimulating an immune response to an HIV antigen in a mammal comprising a recombinant viral construct as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents. The composition may also comprise the recombinant viral construct and a known antiviral compound or molecule.

Further features of the present invention are more fully described in the following non-limiting Examples. It is to be understood, however, that the detailed description is included solely for the purpose of exemplifying the present invention.

EXAMPLE 1

Animals

Macaques (*M. nemestrina*, aged 24-32 months) were free from SRV infection and anaesthetised with Ketamine (10 mg/kg IM) prior to procedures. The studies were approved by the institutional Animal Experimentation and Ethics Committees.

Seven animals were studied that have been previously described [9,10]. Four animals (M2, M3, M4, M5) served as controls and received no vaccines during the course of this study. These 4 animals (M2-5) had been vaccinated with DNA and FPV HIV-1 vaccines (not containing cytokines) 11-19 months prior to study and resisted a HIV-1 challenge 9 months prior to this study. Three animals (M7, M9, M10) had no previous HIV-1 vaccinations and were infected with HIV-1_{LAI} 9 months prior to this study. Two of these animals (M9, M10) received a novel FPV encoding both gag/pol and human IFNγ and one animal (M7) received a FPV vaccine encoding gag/pol only. Each FPV vaccine was given IM into the anterior thigh at 10^8 PFU in 0.3 ml twice, 3 months apart at 9 and 12 months following HIV-1 infection.

All macaques were previously vaccinated with 3 doses of tetanus toxoid (CSL, Parkville, Australia) IM prior to HIV-1 vaccinations. Each animal was assessed twice daily, following vaccination, for visible swelling or redness at the site of injection and for activity by counting a variety of normal macaque behaviours (individual and conspecific play, foraging, displacement, mounting and grooming activities) as previously described [11]. A 25% reduction in total activity compared to the mean baseline activity in the week prior to vaccination was considered significant. Daily temperature recordings were determined by using an electronic hand held thermometer (Braun Thermoscan) and training the animals to have this applied to their tongues for ≥ 1 second. This method of taking temperatures was found to be 0-0.8°C (mean 0.3°C) lower than rectal temperatures taken on sedated macaques using a rectal thermometer on 22 consecutive occasions.

EXAMPLE 2

Vaccines

The HIV-1 gag/pol genes with or without the human IFNγ gene have been inserted into the FPV genome, along with the *E. coli lacZ* and *gpt* marker genes, between the FPV thymidine kinase (TK) region and the 3' open reading frame (ORF). See Figs 1 and 2.

EXAMPLE 3

Blood cell counts and plasma biochemistry

To determine whether the vaccine-expressed IFNγ resulted in abnormalities in plasma biochemistry or blood cell counts, a battery of biochemical and cellular analyses were performed on serial blood samples from the macaques. Multiparameter biochemical analyses and blood counts were performed on automated machines and counts confirmed manually. White cell counts and differential were confirmed by manual counting. PBMC obtained before and after vaccinations were stained for monocyte and T cell markers and analysed by flow cytometry as previously described [9] using antibodies directed against CD2 (Leu5-PE, Becton Dickinson, San Jose, CA), CD4 (OKT4-FITC, Ortho Diagnostics, Raritan, NJ), CD8 (Leu2a-FITC, Becton Dickinson), and CD14.

EXAMPLE 4

HIV-1 Antibody and Th responses

Plasma was assessed for HIV-1 antibodies by particle agglutination (Serodia-HIV, Fujirebio, Japan) and Western blotting using 200μg of standard mixed HIV-1 protein stock [9]. Lymphoproliferative responses were assessed by standard ^{3}-H-thymidine incorporation assay as described [9]. Macaque PBMC in triplicate wells at 10^5 cells/well were stimulated for 6 d with 10μg/ml of recombinant HIV-1_{SF2} gp120 or HIV-1_{SF2}p24 (Chiron) in media containing 10% autologous heat-inactivated serum and pulsed with ^{3}H-

thymidine before β-counting. PBMC were also incubated with media alone or media supplemented with 10μg/ml culture derived control antigens to assess unstimulated control responses, and stimulated with PHA (10μg/ml) or tetanus toxoid antigen (0.01 Lf/ml) as positive mitogenic and antigenic control responses. Proliferation is expressed as stimulation index (SI, mean ^{3}H-thymidine incorporation of cells stimulated with antigen/mean incorporation in absence of antigenic stimulation). Supernatants from selected lymphoproliferative cultures were assayed for the presence of IL-4 and IFNγ by EIA (Genzyme, Cambridge, MA).

EXAMPLE 5

Quantitative HIV-specific CTL analyses

Analysis of CTL precursor frequencies to HIV-1 Env and Gag/Pol antigens in macaque PBMC of macaques was performed by a limiting dilution analysis [9]. PBMC were plated in 96 well round-bottomed plates in 7 serial 1.5-fold dilutions of 10^5 to 8.8 10^3 cells/well in 24 replicates. Each well was stimulated with 10^4 autologous PBMC infected with a recombinant vaccinia virus expressing HIV-1_{LAI}Env/Gag/Pol antigens and supplemented with 10U/ml rIL-2 (Hoffman-La Roche, Nutley, NJ) every 3-4 d. After 10-14 d, cells in each well were assayed for cytolytic activity against autologous target cells infected with wild type vaccinia or recombinant vaccinia expressing HIV-1_{LAI}Env antigens or HIV-1_{LAI}Gag/Pol. Wells were considered positive if cytolysis exceeded the mean spontaneous release from that target by 3 SD. CTL frequencies and 95% confidence intervals were determined by maximum likelihood analysis with software provided by S Kalams, Harvard Medical School [16]. Target cells were autologous B lymphoblastoid cell lines (BLCL), established from each macaque by infecting PBMC with *H. papio*, a baboon herpresvirus [9]. BLCL could not be transformed with PBMC of one control animal (M4) and could not be maintained in long term culture from one vaccinated animal (M10) and CTL data could not be generated from those animals.

EXAMPLE 6

Analysis of HIV-1 DNA and viral levels

HIV-1 *gag* and HLA-DQ DNA were amplified from extracted DNA form PBMC samples and quantified using primer pairs SK38/39 and GH26/27 (Gibco-BRL) respectively using PCR conditions as described [9]. DNA from 10^5 PBMC was standardised according to the DQ band density in comparison to 8E5 cell DNA (which contains 1 HIV-1 DNA copy/cell) and confirmed by measuring absorbance on a spectrophotometer (Ultrospec 3000, Pharmacia Biotech) at 260nm. Virus isolation was performed by cocultivating 10^6 macaque PBMC with 10^6 PHA-stimulated pooled human PBMCs and 50U/ml IL-2. Fresh media and IL-2 were added to the cultures twice weekly and PHA-stimulated human PBMC added weekly for 4 weeks. HIV-1 was quantified in cultures supernatants by HIV-1 p24 EIA (Abbott Laborators, Abbott Park, IL).

EXAMPLE 7

Safety of FPV expressing IFNγ

Locally delivered cytokines encoded by viral vectors are generally less toxic than systemically administered cytokines [8]. We analysed the reactogenicity of FPVgag/pol-IFN-γ in comparison to 4 matched controls not immunized and a control animal receiving FPVgag/pol-IFNγ only. A high dose of the FPV vaccines was administered (10^8 FPU) in an attempt to detect any significant adverse effects. A 44-75% reduction in activity of all 3 FPV-immunised macaques was observed for the first 24 hrs following vaccination, and in one of two FPVgag/pol-IFNγ immunised animal (M9), 28% reduction of activity was present between 24 to 48 hrs, but was normal thereafter in all animals. Swelling at the injection site was observed for 1-2 days following vaccinations in all 3 FPV vaccinated animals. No fever was documented following the FPV vaccinations (Figure 3a). All animals gained weight normally. No change in $CD4^+$ or $CD8^+$ T cell subsets, or monocyte levels in PBMC were observed following vaccination by (Figure 3b). Additionally, no significant changes in plasma electrolytes, renal function as assessed by

plasma creatinine and urea, liver function markers, haemoglobin, white cell counts or platelet counts were observed following FPVgag/pol or FPVgag/pol-IFNγ vaccination.

EXAMPLE 8

T cell immunogenicity

To determine whether vaccination with FPVgag/pol-IFNγ enhanced Gag/pol specific Th responses, macaques infected with HIV-1 9 months previously were vaccinated twice with FPVgag/pol-IFNγ (2 animals, M9 and M10) or FPVgag/pol (1 animal, M7). Th proliferative response to p24 Gag protein was enhanced 4-7 fold 1-2 weeks after the first FPVgag/pol-IFNγ vaccination and was greater than baseline levels 3 months later (Figure 4a). Following a second FPVgag/pol-IFNγ vaccination, p24-specific Th responses were further boosted above baseline (5-30 fold) and maintained for at least a further 2 months. The animal which received 2 FPVgag/pol immunisations had a 3 fold enhancement of p24-specific Th response. Tetanus-specific Th responses did not change following FPV vaccinations (<3 fold variation over time). The Th responses to Gag or tetanus antigens of 4 control macaques (M2, M3, M4, M5) did not change, with a <2 fold variation over the 4 month observation period (means SI to p24 was 3.2 and to Tetanus toxoid 3.6).

We also assessed whether FPVgag/pol-IFNγ vaccination of HIV-1 infected animals was associated with a change in the phenotype of Gag-specific Th response. Enhanced IFNγ secretion, but not IL-4 secretation, by Gag-specific Th responses from PBMC of animals receiving both FPVgag/pol and FPVgag/pol-IFNγ was observed, with the magnitude of the modulation of the cytokine secretion being greater in the FPVgag/pol-IFNγ immunised animals (Figure 4b). No change in the tetanus-specific Th phenotype from animals M7, M9 and M10 was observed following FPV vaccinations, with IL-4 secretion exceeding that of IFNγ (by 4-12 fold) both before and 2-6 weeks after FPV vaccinations of all 3 FPV vaccinated animals.

EXAMPLE 9

HIV-specific CTL activity following FPVgag/pol-IFNγ immunisation

Considerable interest currently focuses on immunisation strategies to maintain CTL responses in the face of marked reduction in antigenic stimulus from replicating HIV-1 [2, 5]. HIV-1 specific CTL response in macaques parallel the reduction in HIV-1 DNA following the first few months of HIV-1 infection, and in the "latent" phase HIV-1 specific CTL responses are low (≤10 HIV specific CTLs/10^6 PBMC) [9]. By a limiting dilution analysis, CTL precursors to Gag/Pol (but not Env) antigens were enhanced from <5 to 15/10^6 PBMC following one FPVgag/pol-IFNγ vaccination and to 44/10^6 PBMC following a second FPVgag/pol-IFNγ vaccination (Figure 3). Gag/Pol or Env specific CTLs were not detectably enhanced (remaining ≤5/10^6 PBMC) in controls animals either unvaccinated (M2, M3, M5) or vaccinated with FPVgag/pol-IFNγ without IFNγ (M7, Figure 5).

EXAMPLE 10

HIV-1 levels following vaccination

To determine whether FPVgag/pol-IFNγ vaccination altered HIV-1 viral levels in macaques previously infected with HIV-1, HIV-1 DNA and culturable virus were studied before and after vaccinations. Using *env*-specific primers, animals M7, M9 and M10 had ≤10 copies of HIV-1 DNA/10^5 PBMC 0, 1 and 4 months prior to vaccinations and remained at ≤10 copies of HIV-1 DNA/10^5 PBMC at 1, 2 and 4 weeks following the first FPV vaccination, without detectable changes in HIV-1 DNA levels. HIV-1 could not be recovered from cocultured PBMC from any of the 3 vaccinated FPV animals either prior to (weeks 0, -4) or following (weeks +1, +2, +4, +6) vaccination. The cocultured method employed has routinely recovered HIV-1 when plasma HIV-1 RNA levels were 100-400 copies [(9); (10)], suggesting a significant rise in HIV-1 plasma RNA did not occur.

EXAMPLE 11

HIV-1 antibody levels

Gag/Pol specific antibodies were also enhanced following the 2 FPV vaccinations (Figure 6). p24-specific antibodies were enhanced in all 3 vaccinated animals, with no difference observed between the FPVgag/pol-IFNγ and FPVgag/pol vaccinated animals. No change in gp120 antibody responses was observed.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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DATED this 9th day of November, 1998

Macfarlane Burnet Centre for Medical Research;
CSIRO and Australian National University
by their Patent Attorneys
DAVIES COLLISON CAVE

Figure 1 Construction of FPV-gag/pol-IFNγ

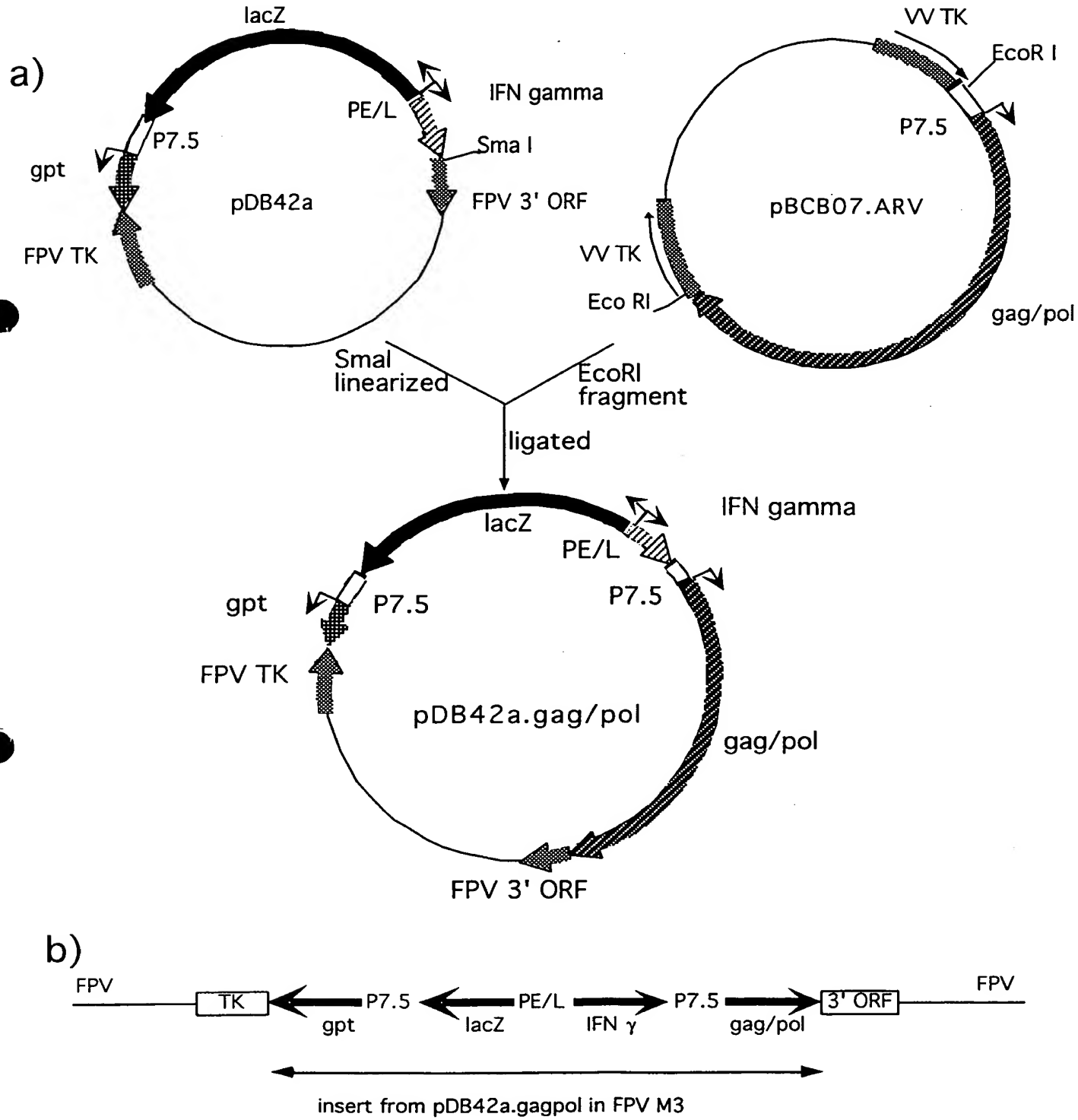
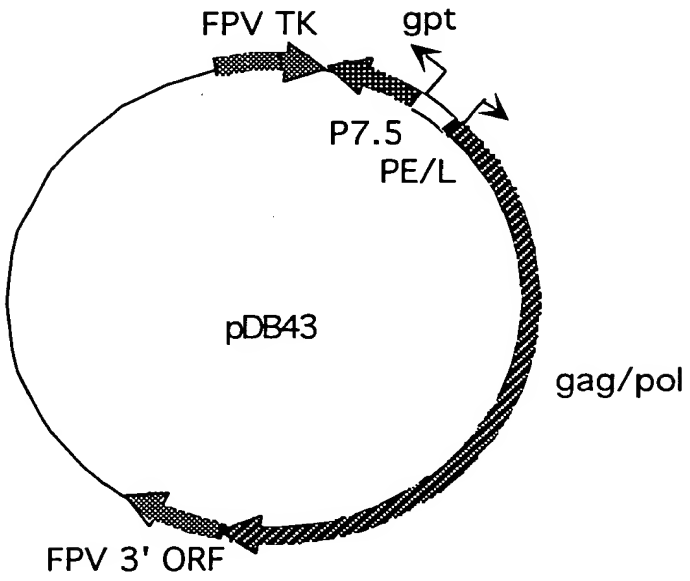


Figure 2: Construction of FPV-gag/pol

a)



b)

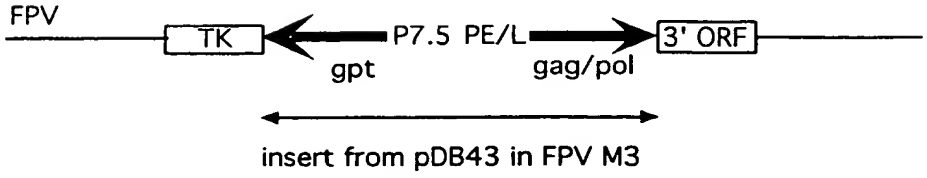


FIGURE 3

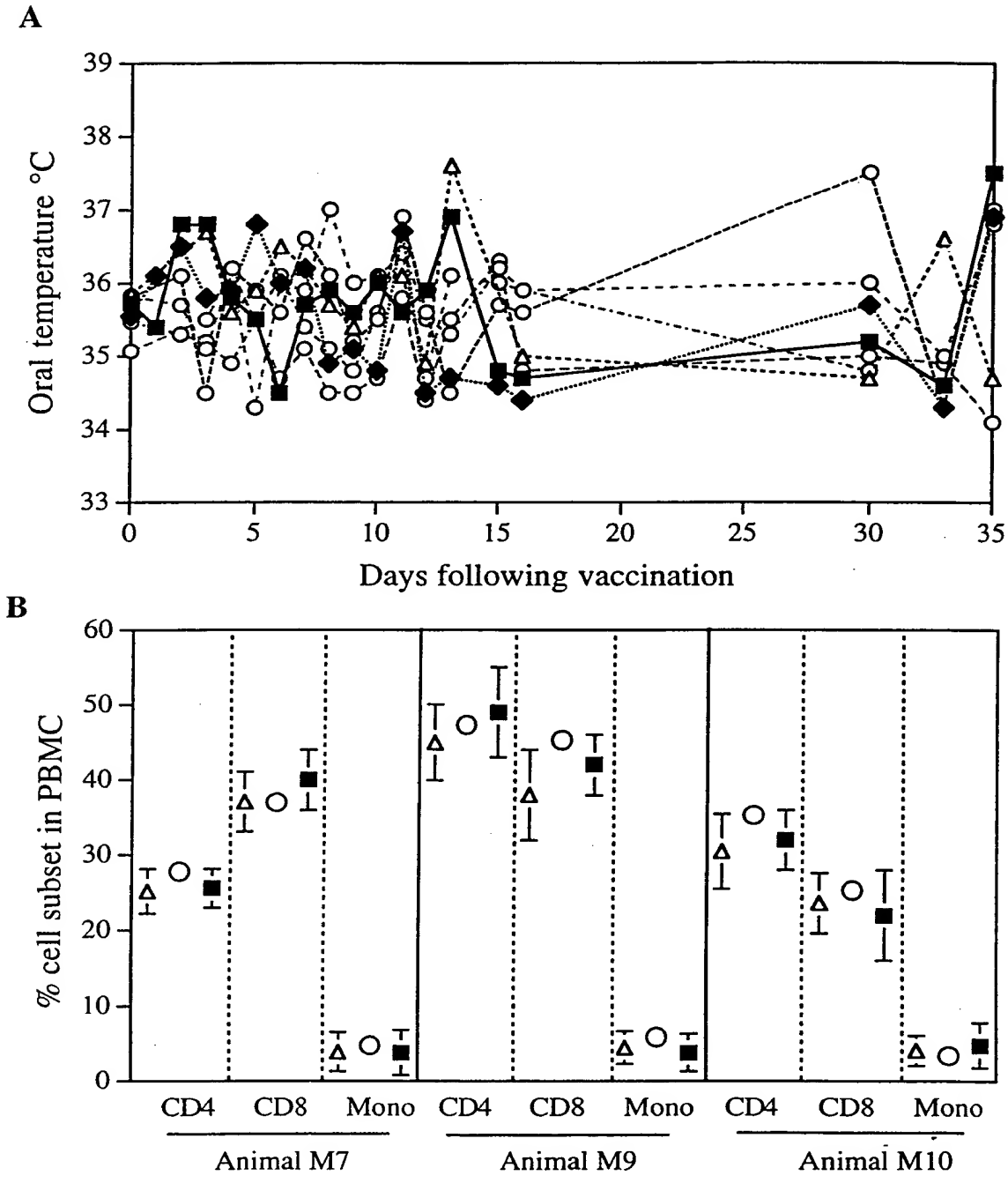

A
Oral temperature °C
39
38
37
36
35
34
33
0
5
10
15
20
25
30
35
Days following vaccination
B
% cell subset in PBMC
60
50
40
30
20
10
0
CD4
CD8
Mono
CD4
CD8
Mono
CD4
CD8
Mono
Animal M7
Animal M9
Animal M10

FIGURE 4

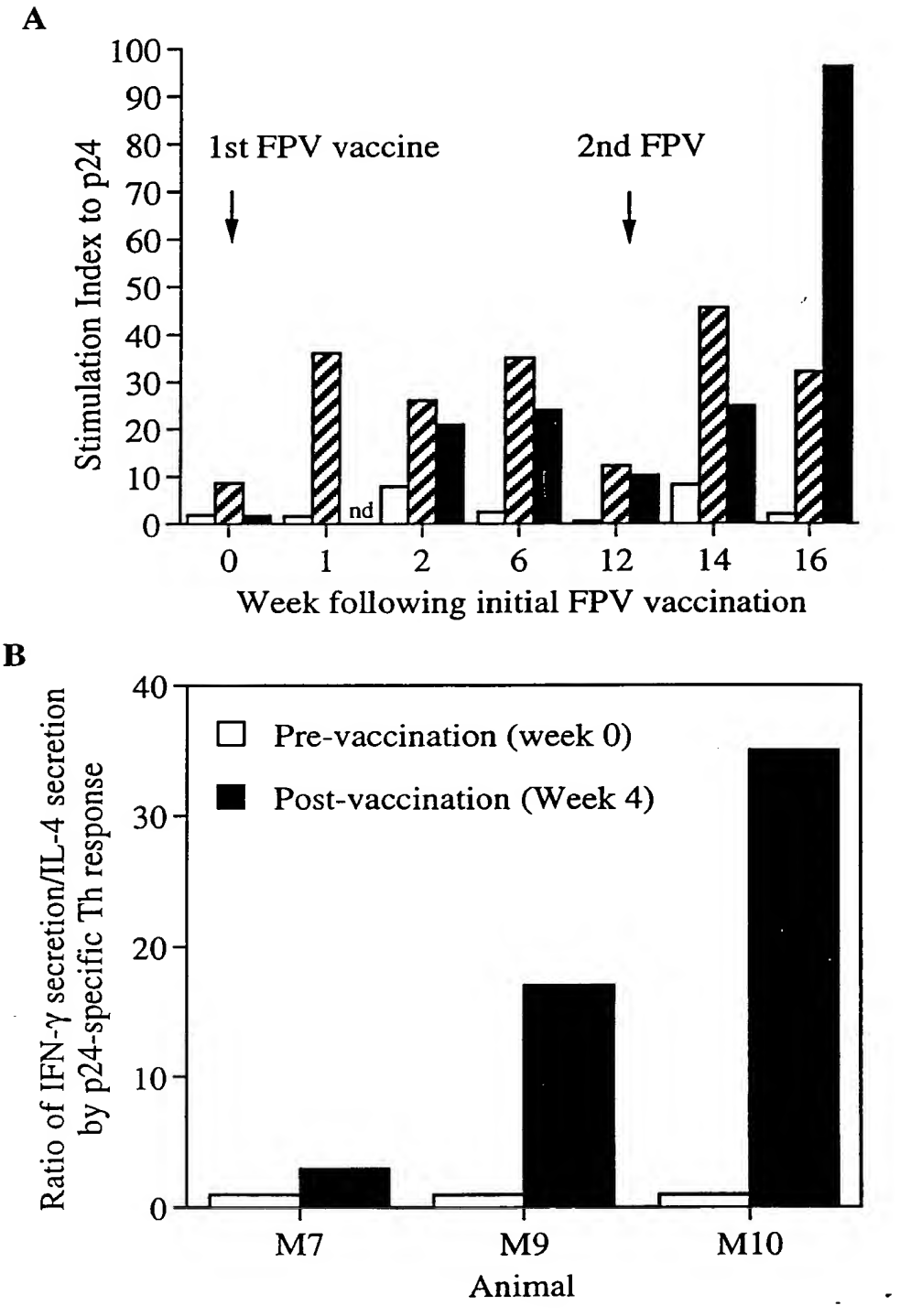

A
100
90
80
70
60
50
40
30
20
10
0
Stimulation Index to p24
1st FPV vaccine
2nd FPV
nd
0
1
2
6
12
14
16
Week following initial FPV vaccination
B
40
30
20
10
0
Ratio of IFN-γ secretion/IL-4 secretion by p24-specific Th response
Pre-vaccination (week 0)
Post-vaccination (Week 4)
M7
M9
M10
Animal

FIGURE 5

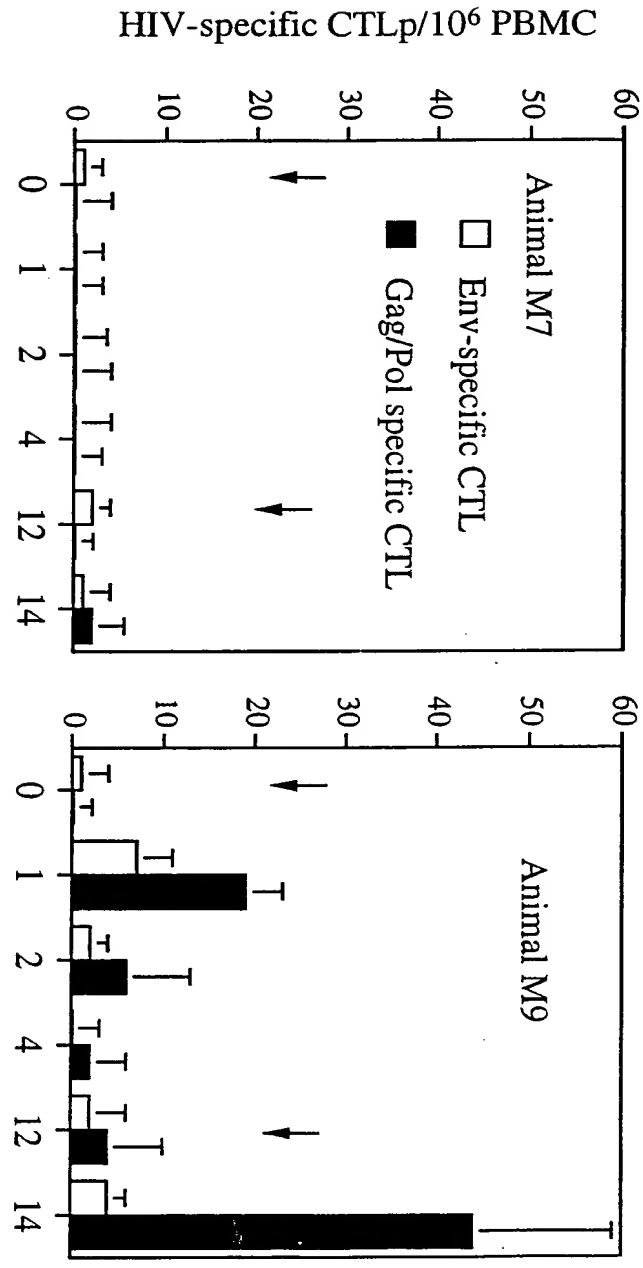
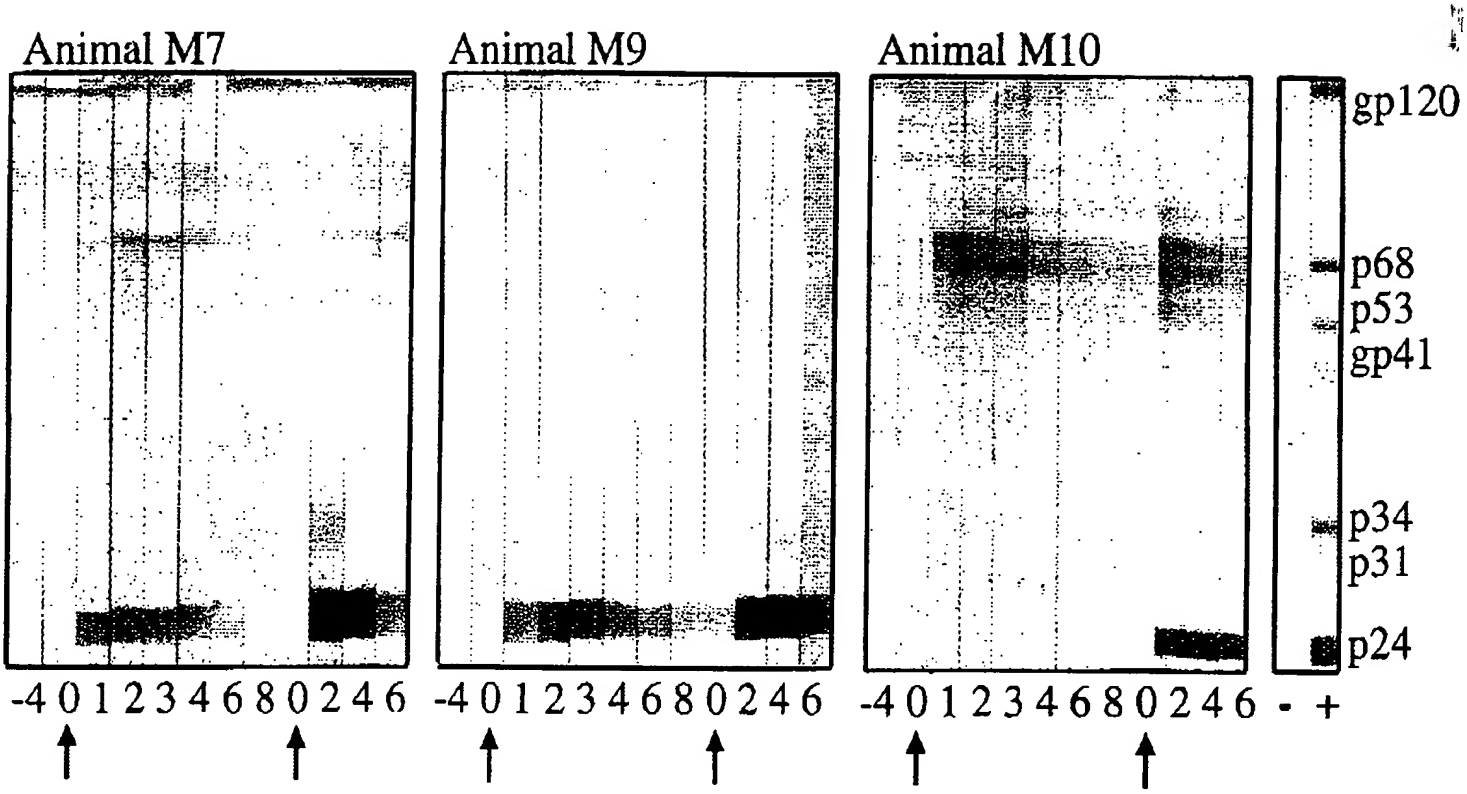

HIV-specific CTLp/10⁶ PBMC
Animal M7
Animal M9
Env-specific CTL
Gag/Pol specific CTL
Week following initial FPV vaccination

FIGURE 6


Animal M7
Animal M9
Animal M10
gp120
p68
p53
gp41
p34
p31
p24
-4 0 1 2 3 4 6 8 0 2 4 6
-4 0 1 2 3 4 6 8 0 2 4 6
-4 0 1 2 3 4 6 8 0 2 4 6
- +